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Analysis of the Effects of Overexpression of Metallothionein-I in Transgenic Mice on the Reproductive Toxicology of Cadmium

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Exposure to low levels of cadmium reduces fertility. In male mice spermatogenesis is highly sensitive to cadmium, whereas in females the peri-implantation period of pregnancy is sensitive. To examine the potential roles of the cadmium-binding protein, metallothionein (MT), in the reproductive toxicology of cadmium, we examined a transgenic mouse strain that overexpresses metallothionein-I (MT-I). These mice had dramatically increased steady-state levels of MT-I mRNA and MT in the testes and in the female reproductive tract during the peri-implantation period of pregnancy, and this overexpression occurred in a cell-specific and temporally regulated manner similar to that of the endogenous *MT-I* gene. Transgenic and control males were injected with cadmium, and the histology of the testes was examined. An injection of 7.5 µmol Cd/kg had no effect on histology of the testes in either transgenic or control mice. In contrast, an injection of 10 µmol Cd/kg caused rapid changes in the histology of the testes and resulted in pronounced testicular necrosis in both control and transgenic mice. Female transgenic and control mice were mated and then injected with cadmium (30–45 µmol Cd/kg) on the day of blastocyst implantation (day 4). In both of these groups, injection of cadmium reduced pregnancy rate, and no dramatic protection was afforded by maternal and/or embryonic overexpression of MT. Thus, overexpression of MT-I does not significantly protect against either of these cadmium-induced effects on fertility. *Key words*: cadmium, metallothionein, peri-implantation, reproductive toxicology, transgenic mice, testes. *Environ Health Perspect* 104:68–76 (1996)

Cadmium is a widely distributed, toxic trace metal. Cadmium poisoning causes damage to major organ systems, and in humans this leads to Itai-Itai disease (*1,2*). This nonessential transition metal is also carcinogenic (*3–5*) and is deleterious to the reproductive process, causing retardation of growth, sterility, teratogenic, and embryotoxic effects (*6–8*). In rodents, spermatogenesis has long been known to be highly susceptible to cadmium. In sensitive strains of mice, the testes undergo necrosis after exposure to cadmium (*9,10*). Likewise, injection of pregnant female mice with a sublethal dose of cadmium on the day of blastocyst implantation (day 4) significantly reduces pregnancy rate by midgestation (*11*). Cadmium exerts embryotoxic effects on preimplantation embryos *in vitro* (*12,13*), and these effects are particularly dependent on the stage of embryonic development, with the blastocyst being the most sensitive to cadmium toxicity (*11,14*). However, cadmium also exerts toxic effects on the female reproductive tract and can delay the onset of implantation (*11*). After implantation, the deciduum, placenta, and visceral yolk sac may serve to protect the embryo by restricting the passage of cadmium from the mother, but exposure to cadmium during the organogenic period can induce limb bud and craniofacial defects (*6,7*). Mechanisms that may protect against the reproductive toxicity of cadmium are largely unknown.

Metallothioneins (MT) are a family of cysteine-rich cadmium-binding proteins (*15*) whose genes are transcriptionally activated by cadmium (*16*). Because MTs have been shown to protect against cadmium toxicity in cultured cells (*17,18*) and in mice (*19–21*) they are prime candidates for protecting against toxic effects of cadmium on reproductive processes.

Several strains of mice that overexpress MT have been created using a minimally mutated *MT-I* (*MT-I**) gene under the control of 1.8-kb of the mouse MT-I promoter and flanked by the MT-I/MT-II locus control regions (LCR) (*22*). In these mice, the *MT-I** transgene is expressed in a copy-number-dependent, integration-site-independent manner, and developmental expression appears to mimic the endogenous gene. We examined one of these transgenic mouse strains and reported that MT is overexpressed in most major organs (*23*). In this study we documented the level of expression and tissue-specific distribution of MT in reproductive organs (uterus and testes) of male and female transgenic and control mice from this strain and evaluated the role of MT in protection against cadmium-induced testicular damage and early pregnancy failure.

Methods

Animals. All experiments involving animals were conducted in accordance with National Institutes of Health standards for the care and use of experimental animals. The transgenic mice used in these studies were derived from heterozygous males that carry 56 copies of minimally mutated MT-I (*MT-I**) gene on the B6/SJL F_1 background (*22*). These mice were outbred to CD-1 females (Charles River Breeding Laboratories, Raleigh, North Carolina), and the heterozygous male offspring were selected. These heterozygous males were bred with CD-1 females, and experiments were performed using the transgenic and nontransgenic (control) littermates from this breeding. We identified transgenic and control mice by DNA slot blot analysis. For experiments involving pregnant animals, virgin females of the indicated strain (48–68 days old) were mated with males of the indicated strain, and the morning a vaginal plug was detected was defined as day 1 of pregnancy.

Cadmium injection. $CdCl_2$ (Sigma Chemical Co., St. Louis, Missouri) was dissolved in acidified normal saline and administered in a single 100-µl subcutaneous injection. Dosages of cadmium ranged from 7.5 to 45 µmol/kg body weight. To determine the effect of cadmium on pregnancy, we injected cadmium on the morning of day 4 and assessed pregnancy success on day 14 as described (*11*). The effect of cadmium on the testes was determined by histological examination 24 and 48 hr after cadmium injection.

MT determination. MT was determined by the cadmium–hemoglobin exchange assay as described (*24*). For convenience, tissue samples were frozen in liquid nitrogen and stored at -70°C before analysis.

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Isolation of RNA and Northern blot hybridization. Tissues were frozen in liquid nitrogen and stored at -70°C before RNA extraction. RNA was prepared by an initial guanidine thiocyanate (GTC)/phenol/chloroform extraction as described (*25*), followed by sodium dodecyl sulfate (SDS)/phenol/chloroform extractions (*26,27*). Poly A^+ RNA was isolated as described by Aviv and Leder (*28*), using total RNA and oligo(dT) cellulose (Collaborative Biochemicals, Bedford, Massachusetts; 20 mg total RNA/0.5 g cellulose).

RNA was size fractionated by formaldehyde-agarose gel electrophoresis and Northern blotted to nylon membranes (*29*). Northern blots were prehybridized, hybridized with a mouse MT-I ^{32}P-labeled cRNA probe (specific activity = 2×10^9 dpm/μg), and washed (*26,29,30*). Hybrids were detected by autoradiography at -70°C with intensifying screens. In all experiments, we stained duplicate gels with acridine orange to verify integrity and equal loading of RNA.

Competitive reverse transcriptase-PCR analysis of MT-I and MT-I* mRNAs. Competitive reverse transcriptase-polymerase chain reaction (RT-PCR) was performed, under reaction conditions essentially as described (*31*), to differentiate between MT-I* and endogenous MT-I mRNAs. Oligonucleotide primers specific for MT-I were designed based on the sequence of the mouse MT-I cDNA that flanks the *MT-I** mutation (*32*):

sense strand
5′ CACCACGACTTCAACGTCCTG 3′
antisense strand
5′ TCTTGCAGGCGCAGGAGCTG 3′

These primers correspond to nucleotides 3–23 in the 5′ untranslated region (exon 1) and to nucleotides 604–623 (exon 2) of the mouse *MT-I* gene, respectively. Total RNA (25 μg) was reverse transcribed with recombinant mouse murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, Maryland) using the antisense oligo as a primer. One-fourth of the RT reaction mixture was amplified by PCR for 20 cycles in a reaction containing 0.2 μM each unlabeled MT-I primer, 1×10^7 cpm of ^{32}P-5′-end labeled antisense MT-I primer ($5–10 \times 10^6$ dpm/pmol), and 1 unit of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut). Cycle parameters were as follows: cycle 1: 94°C for 4 min, primer annealing at 55°C for 2 min and primer extension 72°C for 2 min; cycles 2–20: 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min. RT-PCR products were purified using magic PCR prep columns (Promega Biotech, Madison, Wisconsin) and 1×10^5 cpm was digested with excess restriction enzyme (*Bgl* II and/or *EcoRV*). *Eco*RV will cleave the MT-I* product, whereas *Bgl* II cleaves the endogenous MT-I gene product (*22*). Restriction fragments were separated by 12% nondenaturing polyacrylamide gel electrophoresis. After fixation in 10% acetic acid for 10 min, the gel was dried and exposed to Kodak XAR-5 film at -70°C for a few hours.

Solution hybridization. Oligonucleotide excess solution hybridization was used to quantitate MT-I mRNA levels as described (*33*). Total RNA or poly A^+ RNA (0–30 μg) plus carrier *E. coli* rRNA (20–50 μg; Boehringer Mannheim, Indianapolis, Indiana) were mixed and hybridized with 10 fmol of ^{32}P-end-labeled antisense oligonucleotide complementary to the 3′ untranslated region of MT-I (*34*) as described previously (*30,33*). The oligonucleotide was end-labeled with T4 polynucleotide kinase according to the manufacturer's suggestions (New England Biolabs, Inc., Beverly, Massachusetts) and had a specific activity of $5–10 \times 10^6$ cpm/pmol. After hybridization, S1 nuclease resistant nucleic acids were precipitated with 20% trichloroacetic acid and collected on glass fiber filters (Whatman GF/C; Fisher Scientific, Pittsburgh, Pennsylvania) and the radioactivity was measured by liquid scintillation counting. Samples were in duplicate. Each set of hybridization reactions also included a standard curve generated using known amounts of sense-strand RNA which was transcribed *in vitro* from the MT-I cDNA clone (*33*).

MT antibodies. A rabbit polyclonal antisera against a synthetic peptide corresponding to the amino-terminal 15 amino acids of mouse MT-I (NacMDPNASA STGGSATCamide) (*32*) was prepared commercially by Immuno-Dynamics (La Jolla, California). Fine epitope mapping of rat MT-II suggested that the acetylated amino-terminal pentapeptide is the primary antigenic determinant and that the acetate group on the amino-terminal methionine is important for antigenicity (*35*). Therefore, the peptide was acetylated on the amino-terminal methionine after synthesis and the C-terminal cysteine carboxyl group was converted to an amide. Cysteine residues corresponding to amino acids 5, 7, and 13 were substituted with alanine. For injection, this peptide was conjugated to keyhole limpet hemocyanin through the C-terminal cysteine sulfhydryl group.

Immunohistochemistry and histology. Immunolocalization of MT-I was performed essentially as described (*27,36–38*). Briefly, uteri were excised, cleaned of fat, and either fixed intact in Bouin's solution or implantation sites were grossly dissected from interimplantation regions and then fixed in Bouin's. Testes were removed from mature males (>68 days old) after perfusion fixation with Bouin's and cut into small pieces which were further fixed in Bouin's. Fixed tissues were dehydrated and embedded for paraffin sections. Paraffin sections (7 μm) were incubated in 10% normal goat serum for 10 min before incubation with primary antibody (1:500 dilution) for 16–20 hr at 4°C. For uteri, we used a kit for the anti-rabbit primary antibody (Zymed Laboratories, San Francisco, California) for immunostaining. This kit used a biotinylated secondary antibody, a horseradish peroxidase-streptavidin conjugate, and a substrate chromogen mixture. After incubation in secondary antibody, we blocked endogenous peroxidase by incubation in 0.23% periodic acid in phosphate-buffered saline for 45 sec (*39*). For testis, we used a kit containing a biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, and an AP-red substrate chromogen mixture for immunostaining. After color development, sections were counterstained lightly with hematoxylin, mounted, and examined under bright field. Red deposits indicated sites of immunostaining. No specific staining was detected in control sections incubated without primary antibody or with nonimmune serum. Furthermore, in all cases specific staining was greatly reduced or eliminated in sections in which the primary antibody was neutralized with a 200-fold excess of MT peptide. For histological analysis of the testes, we used the same fixation, embedding, and sectioning procedures, and sections were stained with eosin.

Results

MT-I mRNA Levels

To determine whether MT transgenes are overexpressed in the testes and peri-implantation uterus of transgenic animals, total RNA was prepared from these tissues and MT-I mRNA levels were quantitated by solution hybridization. This assay employed an oligonucleotide that detected both endogenous MT-I and transgene-derived MT-I* transcripts. MT-I mRNA levels were elevated 8.3-fold in total RNA from the testes of transgenic compared with control nontransgenic littermates (Fig. 1A). In the uterus, at sites of embryo implantation, MT-I mRNA levels were elevated 20.2-fold on day 6 and 8.6-fold on day 8 compared to levels in control mice. During this period, days 6–8, there is massive growth of the

deciduum, which surrounds the embryo and actively expresses the entire *MT* gene locus (*33,40*). Northern analysis showed that MT-I mRNA levels in transgenic mice are highest in the implantation sites/deciduum compared to the interimplantation regions of the uterus (Fig. 1B). Solution hybridization and Northern analysis both show a developmental increase in MT-I mRNA levels in the deciduum of control (11.2-fold) and transgenic (4.8-fold) mice between days 6 and 8, as previously shown in CD-1 mice (*40*).

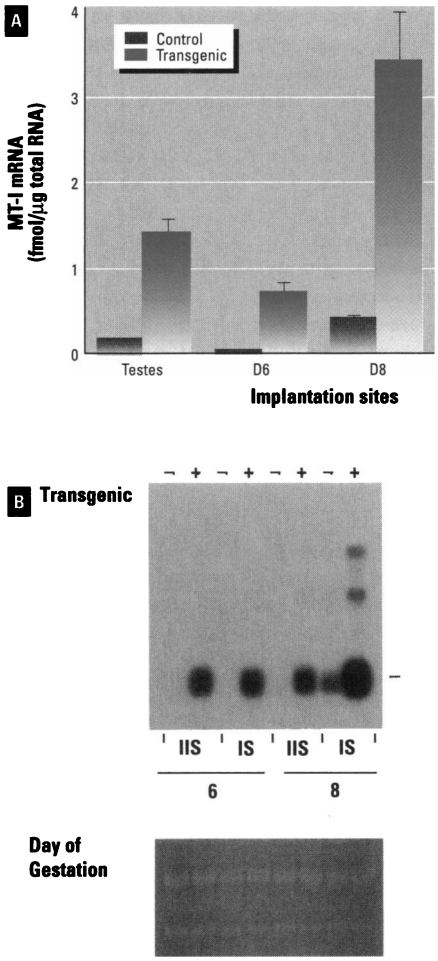


Figure 1. MT-I mRNA levels in the testes and peri-implantation uterus of transgenic and control mice. (A) Total MT-I (MT-I plus MT-I*) mRNA was quantitated by solution hybridization using an excess of an oligonucleotide complementary to MT-I mRNA. Sense-strand MT-I RNA was used to generate a standard curve for quantitation of MT-I mRNA levels. Data are expressed as means ± SDs, $n = 6$). For each tissue, the transgenic mRNA level was significantly different from control ($p < 0.001$, Student's *t*-test). Testis RNA was obtained from four to six animals for each group. Sites of blastocyst implantation site were dissected and pooled from the uteri of pregnant mice ($n = 4$) on days 6 and 8 of pregnancy. Implantation sites on day 8 were dissected free of the myometrium. (B) The top panel is a Northern blot of MT-I mRNA in total RNA (2 µg) from implantation sites (IS) or interimplantation sites (IIS) isolated from the uteri of pregnant mice on days 6 or 8 of gestation/pregnancy. RNAs from control (-) and transgenic (+) mice are compared. The bottom panel is an acridine orange-stained duplicate gel which demonstrates equal loading of 28s and 18s ribosomal RNAs.

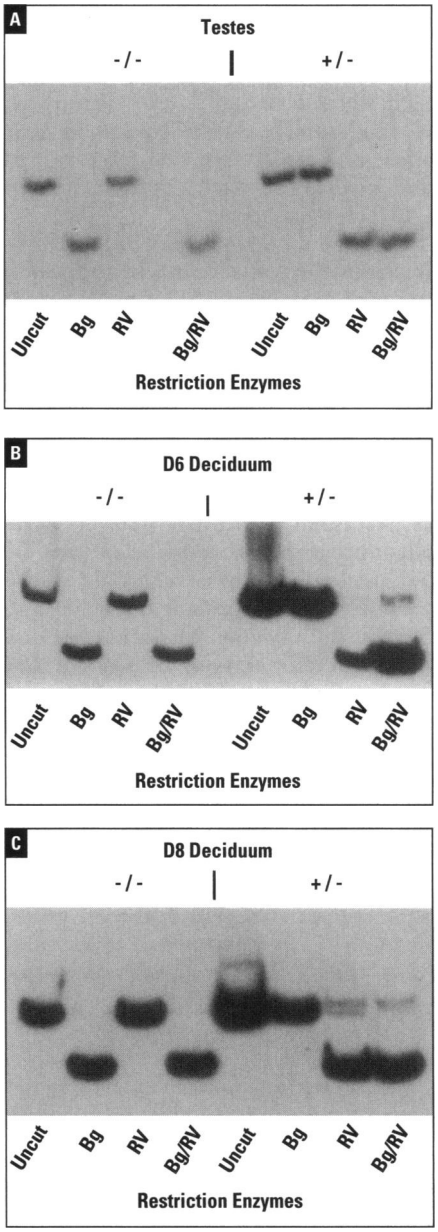


Figure 2. Competitive reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the relative abundance of MT-I and MT-I* mRNAs in the testes and peri-implantation uterus of transgenic and control mice. Total RNA from control (-/-) and transgenic (+/-) tissues was subjected to competitive RT-PCR using oligonucleotides that flank the MT-I* mutation and that are complementary to MT-I and MT-I* mRNAs. The MT-I mRNA RT-PCR product is cut by *Bgl*II (Bg), whereas the MT-I* mRNA RT-PCR product is cut by *Eco*RV (RV). RT-PCR products were radiolabeled by inclusion of a ^{32}P-labeled primer in the PCR reaction. Restriction products were separated by 12% nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography and quantitated by radioanalysis of the gel.

We used competitive RT-PCR to evaluate the relative expression of *MT-I** transgenes and the endogenous *MT-I* gene in the testes and peri-implantation uterus of transgenic mice. Transcripts from the *MT-I** gene contains an *Eco*RV site derived by modification of the *Bgl*II site at +62 in the 5′-untranslated region (*22*). RT-PCR across this region using oligonucleotides complementary to both transcripts, followed by cleavage of the RT-PCR product with *Eco*RV and/or *Bgl*II, differentiates products derived from the *MT-I** transgene and the endogenous *MT-I* gene, respectively. Results of this analysis revealed that RT-PCR products from MT-I* mRNA represent the majority (>90%) of the MT-I mRNA in the testes and peri-implantation uterus from transgenic mice (Fig. 2). As expected, *Eco*RV does not cut RT-PCR products from control mice. These RT-PCR products do not represent PCR of DNA because they are dependent on reverse transcription, and PCR of genomic sequences would yield a larger product due to the inclusion of intronic sequences. The nature of the larger PCR product that was not cut with *Eco*RV or *Bgl*II was not investigated, but probably represents heteroduplexes (*22*).

Determination of MT Levels in Tissues

To determine whether MT accumulates to higher levels in the testes and peri-implantation uterus of transgenic mice, MT was quantitated in heat-stable tissue extracts using the cadmium–hemoglobin exchange assay (*24*). Cadmium-binding was increased significantly in all the major organs examined from transgenic mice compared with those from control mice (Fig. 3), as expected from our previous studies (*23*). In the transgenic testes, MT levels were 5.2-fold higher than in control mice, and in uterine implantation sites MT levels were 8.7-fold higher on day 6 and 2.6-fold higher on day 8 than levels in control mice. As predicted by increases in MT-I mRNA between day 6 and day 8, decidual MT levels correspondingly increased during this period in both transgenic and control mice (Fig. 3). In general, the increases in MT-I mRNA in the transgenic compared to control mice (Fig. 1) are accompanied by increases in heat-stable cadmium-binding activity in these tissues.

Immunohistochemical Localization of MT

Expression of the mouse *MT* genes is cell specific in the peri-implantation uterus (*33,40*) and in the testes (*41*). To deter-

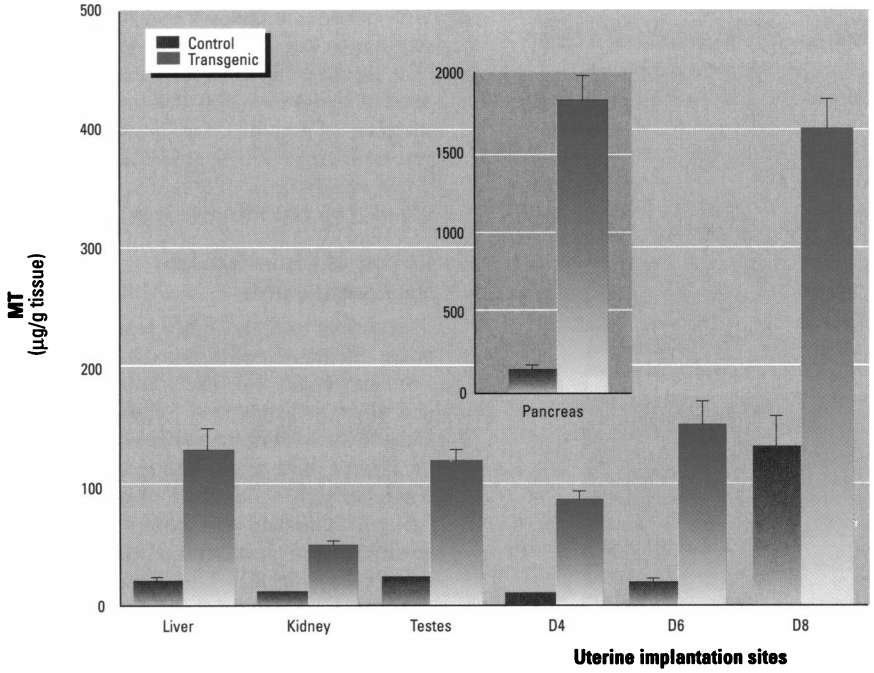


Figure 3. Determination of steady-state levels of MT in tissues of transgenic and control female and male mice. MT was determined by the cadmium–hemoglobin exchange assay (*24*) in heat-stable extracts prepared from the tissues. Uterus, deciduum, liver, kidney, and pancreas were obtained from female transgenic and control mice. Day-4 uterus represents MT in the whole uterus, whereas day-6 uterus represents MT in the implantation sites, and day-8 deciduum represents MT in deciduum dissected free of the myometrium. Testes were isolated from mature breeding males. Data represent the means ± SDs of at least three independent assays of tissues pooled from three animals each. For each tissue the transgenic MT level was significantly different from control ($p < 0.001$, Student's *t*-test).

mine whether the *MT-I** gene is also expressed in a cell-specific manner in these transgenic mice, MT-I was immunolocalized in the day-4 uterus, day-6 and day-8 deciduum, and in the testis (Fig. 4) using an anti-MT-peptide antisera. Our studies of CD-1 mice demonstrated that abundant MT-I mRNA is found in uterine luminal and glandular epithelium on day 4 and in the secondary decidual zone on days 6–8 (*40*). MT-I immunoreactivity was also localized only to these locations in uterine sections from control mice (Fig. 4A). Specificity of the antisera was confirmed using nonimmune sera (not shown) and by antibody neutralization (Fig. 4) before immunostaining. In uterine sections from transgenic mice, MT immunostaining was also restricted to the same cell types as in nontransgenic mice (Fig. 4B), but the immunostaining was notably more intense.

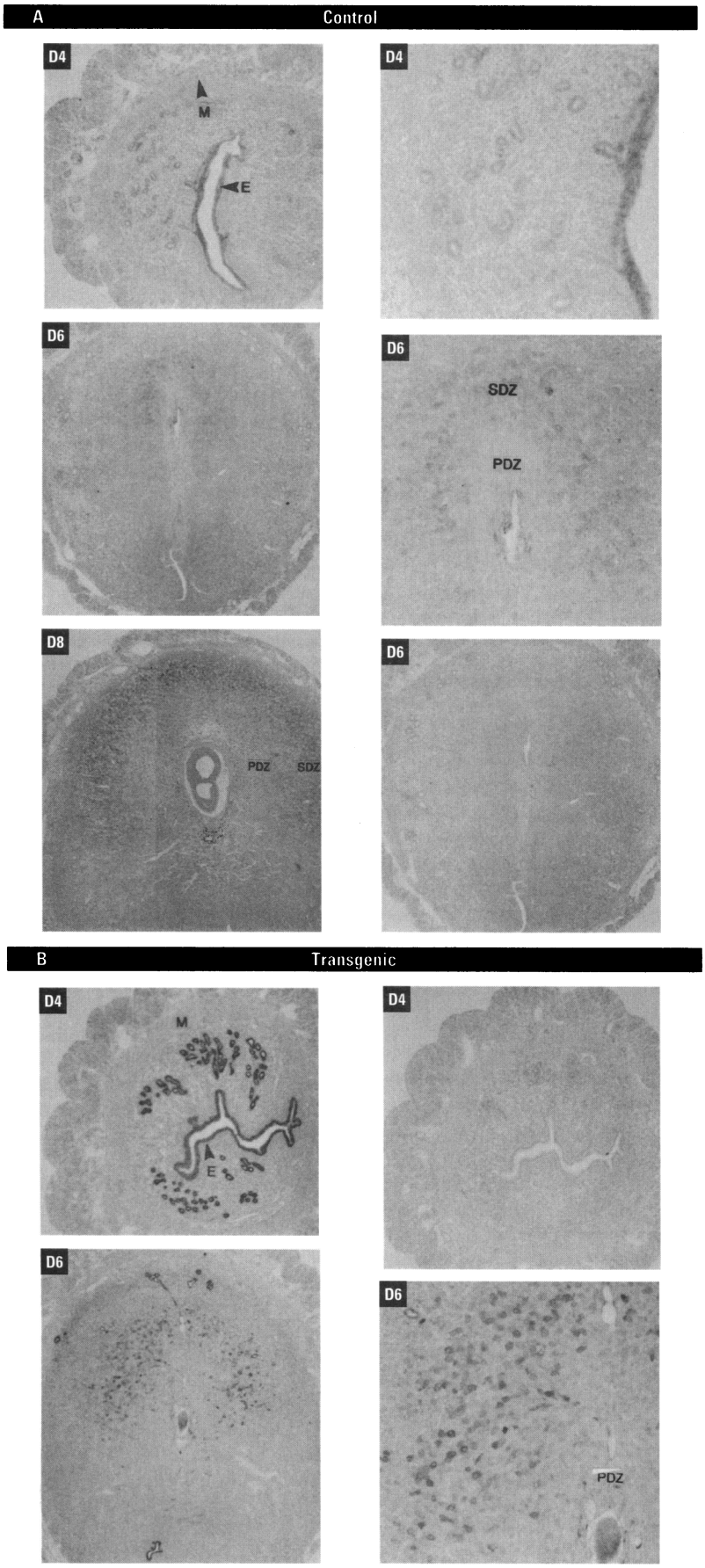
Sections from the testes of control mice displayed only weak MT-I immunoreactivity confined to one or two Leydig cells per field of view (Fig. 5A). Using preabsorbed antibody eliminated this staining (Fig. 5B). In transgenic animals, MT immunoreactivity was also confined to Leydig cells (Fig. 5D, E), but the immunostaining was intense and many Leydig cells were immunopositive for MT. Interestingly, elevated MT-I mRNA levels in the testes reflect accumulation of high levels of these transcripts in the spermatogenic cells (*41*). MT apparently does not accumulate in germ cells, but does so at low levels in Leydig cells in control mice and is elevated in these cells in transgenic mice. The above results establish that the *MT-I** genes are overexpressed, and MT accumulates to higher levels in the proper cell types in the testes and female reproductive tract in this transgenic line of mice.

Effects of Cadmium Injected on the Day of Blastocyst Implantation

Previous studies have documented a marked susceptibility of the blastocyst to acute cadmium exposure both *in vivo* and *in vitro* (*11–13*). We reported that subcutaneous injection of a sublethal dose of cadmium (38 µmol/kg) on day 4 of pregnancy resulted in loss of pregnancy, accompanied by a loss in viability of blastocysts flushed from the uterus on day 5 (*11*). However, maternal effects of cadmium were also noted in those studies and were likely to play a role in the loss of pregnancy. To determine if cell-specific overexpression of MT would protect against cadmium-induced pregnancy failure, transgenic and control mice were given a single subcutaneous injection of cadmium on the morning of day 4 of pregnancy, before blastocyst implantation, and pregnancy was assessed by gross dissection of the uterus on day 14 (Table 1). Mice used in these experiments were divided into three groups based on expression of MT: group 1 mice had normal maternal and embryonic *MT* gene expression and consisted of nontransgenic females (CD-1 or control) mated with CD-1 males. Group 2 mice had normal maternal *MT* gene expression but overexpression of embryonic *MT* and consisted of CD-1 females mated with heterozygous transgenic males (50% transgenic embryos). Group 3 mice had maternal and embryonic overexpression of *MT* and consisted of heterozygous transgenic females mated with CD-1 males (50% transgenic embryos).

In untreated mice, 90–100% of the females that had a vaginal plug on day 1 were found to be pregnant at mid-gestation (day 14), and there were no differences among transgenic, control, and CD-1 females in the number of implantation sites per uterus or the percentage of embryos that had died and were resorbed by day 14 (Table 1). To examine the effects of cadmium on pregnancy, three doses (30, 40, and 45 µmol Cd/kg body weight) were administered on day 4 to mice in each of these groups. Each of these doses of cadmium reduced the pregnancy rate similarly in all three groups of mice. An injection of 30 µmol Cd/kg slightly reduced the pregnancy rate (to 80–90%) in each group, but had no effect on the number of implantation sites per uterus, the day-14 fetal weight, or embryo resorption rate. In contrast, an injection of 40 µmol Cd/kg resulted in a sharp decline in pregnancy rate (46–62%) in all three groups (Table 1). In each experiment the pregnancy rate was consistently higher in mice with maternal and embryonic overexpression of MT (group 3) than in the other groups of mice. However, there were no remarkable differences between the three groups of mice with regard to the effects of cadmium on pregnancy rate.

Under these experimental conditions, 40 µmol Cd/kg was at the low end of the steep dose–response curve for lethality for each of these groups of mice (Table 1). In experiment 1, 40 µmol Cd/kg did not kill any of the mice examined. In contrast, in experiment 2, 1 of 24 transgenic mice died, and 4 of 18 control mice died. An injection of 45 µmol Cd/kg resulted in significant lethality in all groups, but was below the LD_{50}. Surprisingly, there were no remarkable differences in the dose–response curves for cadmium lethality among these groups of mice. It was noted that cadmium injection on day 4 resulted in an all-or-none effect on pregnancy, which is consistent

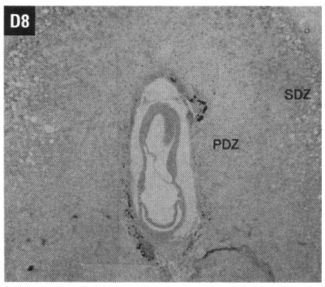


with maternal cadmium toxicity governing pregnancy rate. In all three groups, mice that escaped the effects of cadmium and were pregnant on day 14 had a normal number of implantation sites, and there was no increase in resorption rate (Table 1) Thus, embryonic overexpression of MT afforded no selective advantage.

Effects of Cadmium on Spermatogenesis

The rodent testis is highly sensitive to the toxic effects of cadmium (*9,10,42*), and necrosis occurs after the administration of cadmium at levels well below those that cause liver or kidney damage. Transgenic or control male mice were injected with a single subcutaneous dose of cadmium at 7.5 or 10 µmol/kg, and 24 hr or 48 hr later the testes were recovered and examined histologically (Fig. 6). The histology of the testis 48 hr after an injection of 7.5 µmol Cd/kg was unchanged in both transgenic and control mice (Fig. 6A,B). However, 24 hr after an injection of 10 µmol Cd/kg, alterations in the seminiferous epithelium were apparent (Fig. 6C,D) and by 48 hr extensive testicular necrosis, edema, and sloughing of the seminiferous epithelium had occurred in both transgenic and control mice (Fig. 6E,F). These results demonstrate that overexpression of *MT* affords no apparent protection against cadmium-induced testicular necrosis.

Figure 4. Immunohistochemical localization of MT in the peri-implantation uterus from transgenic and control mice. Control (A) and (B) transgenic mice were mated, and uteri were removed on day 4, or implantation sites were dissected from uteri on days 6 and 8. See Methods for details. Dark deposits show MT immunostaining. Preabsorption of the antisera with MT peptide eliminated immunostaining (A, bottom right; B, top right). E, epithelium (luminal and glandular); M, myometrium; PDZ, primary decidual zone; SDZ, secondary decidual zone. (A) (top left) day-4 uterus, 28×; (top right) day-4 uterus, 69×; (middle left) day-6 implantation site 28X; (middle right) day-6 implantation site, 69×; (bottom left) day 8 implantation site, 21×; (bottom right) day-6 preabsorbed antisera control, 28×. (B) (top left) day-4 uterus, and (top right) preabsorbed antisera control, both 28×; (bottom left) day 6 implantation site, 28X; (bottom right) day-6 implantation site, 69×; (directly below) day-8 implantation site, 25×.



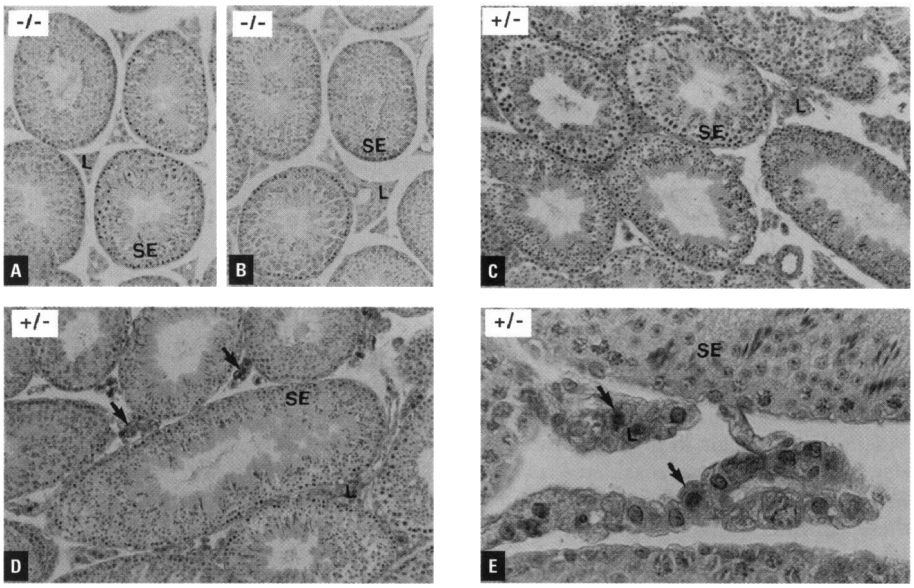


Figure 5. Immunohistochemical localization of MT in the testis of transgenic and control mice. Testes were recovered from (A,B) control (-/-) and (C–E) transgenic (+/-) males. See Methods for details. Dark deposits show MT immunostaining. Preabsorption of the antisera with peptide eliminated immunostaining (B,C). SE, seminiferous epithelium; L, Leydig cell. Panels (A,B) 62×, (C,D) 69×, (E) 276×. Arrows indicate MT immunostaining in Leydig cells. In panel A, control testes immunostaining was confined to one cell in this field of view, and the staining was faint, making it difficult to reproduce photographically.

Table 1. Effects of cadmium injection on day 4 of pregnancy in transgenic, control, and CD-1 mice[a]

	Parents		Cd	*n* (total	Lethality	Pregnancy rate	Embryos per	Embryos resorbed	Embryo weight
	Female	Male	(μmol/kg)	female)	(%)[b]	(%)[c]	female[d]	(%)	(g)
Experiment 1	CD-1	CD-1	30	10	0	80	10.9 ± 1.9	3.7	0.13
			40	14	0	50	11.2 ± 2.2	6.9	
	CD-1	Transgenic	30	9	0	89	12.0 ± 1.8	6.2	0.12
			40	13	0	46	11.8 ± 1.2	4.4	
	Transgenic	CD-1	30	11	0	82	9.0 ± 3.5	6.0	0.13
			40	8	0	62	10.2 ± 1.8	7.8	
Experiment 2	CD-1	Transgenic	40	13	0	54	13.1 ± 2.2		
			45	13	23	50	14.4 ± 1.3		
	Transgenic	CD-1	40	24	4	60	10.3 ± 4.5		
			45	8	38	57	10.2 ± 3.0		
	Control	CD-1	40	18	22	57	12.0 ± 1.4		
			45	10	20	50	10.8 ± 1.9		
Untreated	CD-1	CD-1	0	4	—	100	13.2 ± 1.5	3.8	
	Transgenic	CD-1	0	20	—	90	10.5 ± 2.4	6.0	
	Control	CD-1	0	11	—	100	12.4 ±1.8	5.3	

[a]Female and male mice of the indicated lines were mated, and on day 4 (day 1 = vaginal plug) females were administered the indicated dosage of $CdCl_2$ in a single 100 μl subcutaneous injection. On day 14 animals were sacrificed and pregnancy status was evaluated after gross dissection.
[b]Determined as the number of animals that died between day 4 and day 14 of pregnancy, inclusive, divided by the total number of animals injected with cadmium in that group.
[c]Determined as the number of day 14 pregnant animals divided by the total number of mice alive on day 14.
[d]Data represent the means ± SDs.

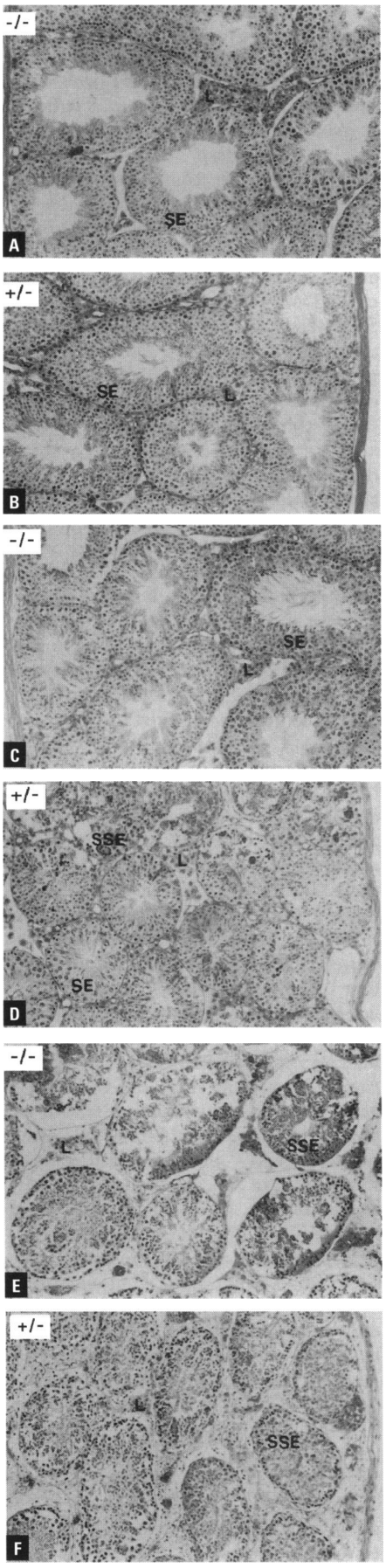
Discussion

The transgenic mice used in this study have stable integration of 56 copies of an *MT-I** transgene with 1.8 kb of the MT-I promoter and flanked by 10 kb of DNA 5′ of the *MT-II* gene and 7 kb of DNA 3′ of the *MT-I* gene (*22*). This construct is expressed in a manner that resembles the endogenous *MT-I* gene (*22,23,43*). We extended those studies to include the peri-implantation uterus where the temporal as well as spatial patterns of expression of the endogenous *MT-I* gene (*40*) and the *MT-I** transgene were indistinguishable. This suggests that the *MT-I** transgene-LCR construct contains all of the information necessary to direct developmental expression of the *MT-I* gene in the uterine epithelium and the deciduum. Although the DNA regions involved in this developmental regulation are unknown, we recently reported that the entire *MT* gene locus is activated in the mouse deciduum (*33*). This is the only known tissue in which each of the four mouse *MT* genes are activated, which suggests that the chromatin domain structure of the *MT* locus may be particularly important in regulating expression in these cells.

This transgenic mouse strain provided a useful tool to examine the functional significance of MT in cadmium toxicity. Specifically, effects of MT overexpression on cadmium toxicity during early pregnancy and on the testes were examined. Surprisingly, these indicators did not reveal a remarkable protective effect of overexpression of MT, although a slight protection from loss of pregnancy was noted. Our previous studies of these transgenic mice revealed that they are more resistant to cadmium-induced hepatotoxicity and acute lethality compared with control mice (*19*), but the dose–response curves for cadmium-induced lethality revealed only a subtle increase (1.2-fold) in cadmium dosage resulted in equivalent lethality between the transgenic and control mice. Such subtle differences in dose response to cadmium were not revealed in the limited dose–response experiments here.

By comparison, cultured cells that overexpress MT, after transfection with an MT expression vector, can resist 12- to 15-fold higher concentrations of cadmium in the culture medium than control cells (*18*), and cultured cells selected for cadmium resistance that overexpress MT can resist up to 60-fold higher concentrations of cadmium (*44*). Interestingly, primary hepatocytes from this transgenic strain that overexpresses MT are about 7-fold more resistant to cadmium toxicity *in vitro*. Thus, large differences between transgenic and control mice in their resistance to cadmium toxicity were predicted based on studies of cultured cells, but studies of these transgenic mice demonstrate that within the physiological context of the animal, overexpression of MT affords little protection from cadmium toxicity.

In contrast, studies of mice in which the *MT-I* and *MT-II* genes have been disrupted suggest a significant increase in sensitivity to cadmium-induced hepatotoxicity and lethality (*20,21*). Although no detailed dose–response curves for cadmium toxicity have been reported, studies of these mice reveal clear increases in cadmium toxicity. Control mice can tolerate 14 daily injections of 10 μmol Cd/kg, whereas the knockout mice succumb after 2–4 days, depending on sex (*21*). Embryonic fibroblasts from these knockout mice are also 2- to 3-fold more sensitive to cadmium *in vitro* than are control fibroblasts (*45*).



Therefore, MT can serve a protective function against cadmium *in vivo*, but overexpression of MT in a proper cell-specific manner does not significantly augment that protective function.

It is perhaps surprising that overexpression of MT at its normal sites of synthesis in the mouse did not effectively protect early pregnancy or spermatogenesis from cadmium toxicity by scavenging cadmium from the serum. After acute exposure to cadmium, the liver can sequester up to 60% of the cadmium challenge (*46,47*), and hepatotoxicity is closely associated with cadmium-induced mortality (*48,49*). MT in the transgenic liver was significantly elevated, and the liver is considered responsible for cadmium detoxification. Despite these considerations, no protection was afforded these reproductive processes, and low levels of cadmium caused testicular necrosis in both the control and transgenic mice.

In the testes of nontransgenic animals, MT immunostaining using an antipeptide antisera was confined to a few Leydig cells. In transgenic animals, MT immunostaining was exclusively in Leydig cells, but was much more intense. These results suggest that the Leydig cell is the only cell that accumulates immunodetectable MT in the mouse testes and that levels of MT are normally low in the control testes. Previous studies have yielded conflicting results regarding the immunolocalization of MT in the rodent testes. MT has been shown to be immunolocalized to Leydig cells in the mouse testis (*50*), but in the rat testes MT immunostaining in Sertoli cells and interstitial cells has been reported (*51*), as has localization in spermatogenic cells, but not in interstitial cells (*52*). It is interesting that MT immunoreactivity was not present in mouse germ cells, as pachytene spermatocytes and round spermatids express high levels of MT-I mRNA and can synthesize MT (*41*). Pachytene spermatocytes and round spermatids isolated from these transgenic mice also contained high levels of MT-I mRNA, and the vast majority of it was derived from the *MT-I** transgene (data not shown). The lack of MT immunostaining in these germ cells is consistent with the suggestion that MT accumulation is controlled primarily by post-transcriptional mechanisms in male germ cells (*41*).

Figure 6. Effects of cadmium injection on histology of the testes from transgenic and control mice. (A,C,E) Control (-/-) and (B,D,F) transgenic male littermates were given a single injection of 7.5 µmol Cd/kg (A,B) or 10 µmol Cd/kg (C–F), and the testes were recovered 24 hr (C,D) or 48 hr (A,B,E,F) after the injection. Testes were fixed in Bouin's solution, and paraffin sections were prepared and stained with hematoxylin and eosin. SE, seminiferous epithelium; L, Leydig cell; SSE, sloughing seminiferous epithelium. (A–F) 39×.

Some inbred strains of mice show remarkable resistance to testicular necrosis produced by lethal doses of cadmium (*53,54*), and this is attributed to the autosomal recessive *cdm* gene (*55*). Although it has been proposed that in the rat, testicular susceptibility to cadmium is the result of the low expression of *MT* genes (*56,57*), the studies reported here suggest that the *cdm* gene is a far more important genetic determinant for testicular sensitivity to cadmium than is the level of testicular MT. Overexpression of MT in the testes was not sufficient to protect from cadmium-induced testicular damage. Leydig cells are not required for the effects of cadmium on the testes (*58*), but elevation of MT in Leydig cells present in the interstitium of the testes might be expected to protect the germ cells because the interstitium is the major site of cadmium accumulation in the testes (*59,60*). Collectively, these studies establish that MT in the interstitium is of limited use in preventing testicular injury after cadmium exposure.

Our earlier studies indicated that pregnancy failure after cadmium exposure on day 4 may have resulted from a direct embryotoxic effect on the blastocyst (*11*). However, the results obtained here, using a larger number of mice, strongly suggest that effects of cadmium on the mother play the key role in the maintenance of pregnancy. Although transgenic blastocysts from these mice display marked elevation in MT (approximately 10-fold) compared to control blastocysts and more resistant to Cd toxicity *in vitro* (*43*), studies reported here establish that transgenic embryos have no selective advantage *in vivo* after maternal exposure to cadmium under these experimental conditions. Pregnancy failure after cadmium exposure on day 4 may result from effects on the uterus; for example, on uterine receptivity for implantation. We previously reported that cadmium can cause a delay in the initiation of implantation (localized sites of increased uterine vascular permeability) in mice (*11*). After implantation, cadmium has restricted access to the embryo, perhaps due to the high levels of MT expressed in the deciduum and visceral yolk sac (*6,7,61*). Despite increased expression of MT in these tissues, the transgenic mice were not significantly protected from cadmium toxicity during the peri-implantation period.

Studies of cadmium toxicity in these transgenic mice that overexpress MT are consistent with the likelihood that cadmi-

um exerts its lethal effects, in part, on cells that do not overexpress MT. It has been suggested that hepatic endothelial cells are a target of cadmium toxicity and that this may account for strain differences in susceptibility (*62*). Cadmium-induced testicular damage has also been suggested to result from injury to the vasculature, which leads to ischemia and death of seminiferous epithelium (*63–65*). In that regard, the process of blastocyst implantation involves highly localized increases in vascular permeability at the sites of embryo implantation. Immunolocalization of MT in these transgenic mice did not suggest enhanced accumulation in endothelial cells of the testes or uterus. The ovary and placenta are also suceptible to cadmium toxicity (*6*), but dosages of cadmium that disrupt the testes had little effect on the establishment and maintenance of pregnancy.

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